International Immunopharmacology 63 (2018) 270–281

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Mimetic peptide AC2-26 of annexin A1 as a potential therapeutic agent to treat COPD

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ARTICLE INFO

Keywords:
Annexin A1
COPD
Inflammation
BAL
FPR2

ABSTRACT

Chronic obstructive pulmonary disease (COPD) is related to inflammatory process caused by smoking habit. In this scenario, the anti-inflammatory protein Annexin A1 (AnxA1) may represent a therapeutic alternative. We performed experiments to evaluate the effects of the AnxA1 mimetic peptide Ac2-26 in an initial COPD model by physiological, histopathological, biochemical and immunohistochemical analyses. Weight loss, increased blood pressure, reductions in the pulmonary frequency and ventilation, loss of tracheal cilia, enlargement of the pulmonary intra-alveolar spaces and lymphoid tissue found in untreated smoke-exposed group were attenuated by AnxA1 peptide treatment. The Ac2-26 administration also protected against leukocytes influx in bronchoalveolar lavage (BAL), lung and trachea, and it also led to decreased hemoglobin, glucose, cholesterol, gamma glutamyl transferase and aspartato aminotransferase levels. Similarly, reduction of proinflammatory mediators and higher concentration of anti-inflammatory cytokine were found in macerated lung supernatant, blood plasma and BAL in the treated animals. Besides Ac2-26 group showed reduced tissue expressions of AnxA1, cyclooxygenase-2 and metalloproteinase-9, but formylated peptide receptor 2 (FPR2) overexpression. Our results all together highlighted the protective role of the Ac2-26 mimetic peptide in COPD with promising perspectives.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a serious health condition estimated to be the third cause of death in 2020 [1,2]. The disease is characterized by progressive limitation of airflow and can be associated with the smoking habit [2,3]. Although COPD has a high incidence in men worldwide, the number of affected women has considerably increased at an alarming rate. In addition, women may respond to tobacco exposure with augmented oxidative stress, enhanced risk of airflow obstruction and impairment of lung function compared to men [4–7].

COPD is presented according to the progressive intensity of the smoking habit [8]. However, in animal models, it has been reported that the reduction in the time of exposure to tobacco associated with increasing of the cigarettes number per day produces similar results to those found by long-term exposure [9–11]. In a rat model of COPD [9], after 4 weeks of tobacco smoke exposure for 6 h a day and 3 days a week, signs of alveolar damage and the presence of neutrophils within the lung parenchyma were observed. The significant increases in airspace enlargement after 4 and 12 weeks of tobacco smoke exposure were similar, and an increase in total leukocytes recovered from bronchoalveolar lavage (BAL) was also showed after 4 weeks of tobacco

Abbreviations: μL, Microliter; μm, Micrometer; Ac2-26, Mimetic peptide Ac2-26 of annexin protein; AnxA1, Annexin A1 Protein; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; BAL, Bronchoalveolar lavage; BALT, Bronchus associated lymphoid tissue; COPD, Chronic obstructive pulmonary disease; COX-2, Cyclooxygenase 2; CS, Cigarette smoke; CS + Ac2-26, Cigarette smoke with mimetic peptide Ac2-26; ED-1, Antibody macrophage marker; FPR, Formylated peptide receptor; G, Gram; Gamma GT, Gamma Glutamyl Transferase; HE, Hematoxylin-Eosin; HRP, Horseradish peroxidase; IL-1β, Interleukin-1beta; IL-6, Interleukin-6; IL-10, Interleukin-10; I.P., Intraperitoneal; L, Liter; LDL, Low density lipoprotein; LPS, Lipopolysaccharide; MCP-1, Monocyte chemotactic protein-1; Mg, Milligram; mL, Mililiter; mm, Milimiter; MMP-9, Matrix metalloproteinase −9; NF-κB, nuclear factor κB; P, Value of P (significance of the statistical test); PBS, Phosphate buffered solution; Pg, Picogram; RPM, Rotation per minute; S.E.M, Standard error of mean = Standard error of mean; TNF-α, Tumor necrosis factor-alpha; U/L, Ultra/liter; Vs, Versus

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https://doi.org/10.1016/j.intimp.2018.08.011
Received 14 March 2018; Received in revised form 8 August 2018; Accepted 10 August 2018
Available online 16 August 2018

smoke exposure [9]. Other research using a rat-COPD model [10] indicated that daily cigarette smoke exposure for 2 h during 5 weeks promoted respiratory lesions similar to those observed in the 13 weeks exposure time. Reduced body weight and significant increases in neutrophil counts were also found after 5-weeks of exposure [10].

The inflammatory process induced by the inhalation of noxious particles and gases leads to pathological alterations, including mucosal hypersecretion, structural changes in the airways and loss of alveoli [2,3].

In the development of the disease, macrophages and respiratory tract epithelial cells activated by inhaled irritants release chemical mediators [2,12] that attract leukocytes and mast cells into the airways [13]. The selectin family adhesion molecules, as L-selectin on the neutrophil surface and E- and P-selectins on the endothelial cell surface are related to cigarette smoke-induced attachment of polymorphonuclear cells to endothelial cells [14].

In addition, airway dysfunction by smoking is associated with high expression of cytokines released especially by mast cells [15]. The number of inflammatory cells in bronchial biopsies and induced sputum can be correlated with disease seriousness as well as lung function and health condition declines. The inflammatory mediators also increase in disease exacerbation [3]. Tumor necrosis factor (TNF)-α and interleukin (IL)-1β are also important in neutrophil margination and attachment to endothelial cells by stimulating the production of adhesion molecules [14]. The increase in monocyte chemokine protein (MCP)-1 levels and a reduction in anti-inflammatory cytokines may also be involved in the inflammatory process in smokers with asthma or COPD [16,17]. Besides, studies have shown that levels of inflammation serum biomarkers, including matrix metalloproteinase (MMP)-9 are higher in COPD patients and linked to the degree of airflow obstruction and mortality [18].

The central role played by inflammation in COPD indicates that the development of novel anti-inflammatory therapies is critical, in particular to slow down disease progression and ameliorate the control of exacerbations [3,19]. Among the anti-inflammatory mediators, the endogenous protein Annexin A1 (AnxA1), the first characterized member of the annexin superfamily [20–22], may represent an alternative therapy for the treatment of COPD and other diseases caused by smoking. AnxA1 is a calcium-dependent binding protein of 37 kDa that also binds to membrane phospholipids and is involved in the inhibition of eicosanoids and cytosolic phospholipase A2 syntheses induced by glucocorticoid [23–25]. Structurally, annexins comprise two domains, a small N-terminal region, which varies in length and composition, and a highly conserved C-central domain. The N-terminal domain is unique to each member of the superfamily, it confers the specific activities and functions of annexins and contains sites for post-translational processes, such as phosphorylation, glycosylation, and proteolysis [20,25–27].

In the inactive cells, AnxA1 predominantly has intracellular location, being translocated, after activation, to the cell surface where it interacts with G protein coupled transmembrane receptors, the formylated peptides receptors (FPRs) [20,27]. Studies indicate that the FPR2 receptor is present in activated lung epithelial and inflammatory cells being particularly important for the COPD resolution [21].

After discovering that the biological activity of AnxA1 could be reproduced by the first amino acids of the N-terminal portion of the protein (peptide Ac2-26), it became common practice to use these molecules in experimental models of inflammation [27–31]. Intravital microscopy analysis of inflamed vessels in vivo pointed the site of action of AnxA1 in adherent leukocytes and the administration of exogenous AnxA1 or its derived peptide, Ac2-26, reduced adhesion and migration of leukocytes in the endothelium of post-capillary venules [26]. Moreover, leukocyte detachment appears to be mediated by the shedding of L-selectin on the surface of inflammatory cells [23–25].

AnxA1 protein is strongly expressed in alveolar macrophages and in human and animal airway epithelial cells [28]. The impact of AnxA1 as a mediator in the control of inflammatory and fibrotic phases was studied in a model of bleomycin-induced pulmonary fibrosis using knockout mice for AnxA1 [29]. The absence of the protein caused increased inflammation degree and fibrosis rates as well as higher transforming growth factor (TGF)-β1, interferon (IFN)-ɤ and TNF-α levels. However, treatment with the mimetic peptide of AnxA1 reduced the signs of inflammation and fibrosis [28]. This protective effect of Ac2-26 was also investigated in a model of pulmonary endotoxemic by local or systemic administration of lipopolysaccharide (LPS) [29]. Pretreatment with the peptide was able to reduce leukocyte influx and proinflammatory cytokines while it increased the anti-inflammatory mediator IL-1β to blood plasma. Other research also showed altered lung functions and exacerbated inflammatory and fibrotic responses on AnxA1 knockout mice exposed to silica [31]. Besides, it was suggested that he impaired synthesis or degradation of AnxA1 may influence immune responses in animals exposed to cigarette smoke through T helper cells [13].

In view of the above, we performed the administration of the AnxA1 mimetic peptide, Ac2-26, in a cigarette smoke model as a possible therapeutic alternative in the management of COPD.

2. Material and methods

2.1. Animals

Female Wistar rats (n = 30), 6 weeks old, were obtained from the Didactic and Experimental Research Unit of University Center Padre Albino, in Catanduva,-São Paulo, Brazil. The animals were divided into 3 groups (n = 10/group): control, exposed to compressed air only (control), exposed to smoke without treatment (CS) and exposed to smoke and treated with Ac2-26 peptide (CS + Ac2-26). The rats were kept in cages in a temperature controlled environment (22 to 25 °C) with water and food ad libitum. All experimental procedures were conducted according to the guidelines for biomedical research stated by the Brazilian Societies of Experimental Biology and approved by the Ethic Committee on Animal Use at University Center Padre Albino (Certificate n° 01/15). The experiments were designed to minimize the number of animals used and their suffering during the execution of the protocols. All animals were daily evaluated by the institution's veterinarian.

2.2. Exposure to cigarette smoke and treatment with Ac2-26 mimetic peptide protocols

Two groups of animals were induced to initial COPD in a specific smoke exposure apparatus. The equipment consists of an animal containment system and a cigarette smoke release system with an external cigarette holder connected to a dynamic suction pump. The pump can be programmed so that cigarette suction periods alternate with periods of clean air suction to prevent asphyxiation [32,33] (Supplementary Video 1). The exposures were standardized and performed twice a day using commercial cigarettes (containing 0.8 mg of nicotine, 10 mg of tar and 10 mg of carbon monoxide). The animals were exposed to the burning of 10 cigarettes in the morning (7 a.m) and 10 cigarettes in the early evening (6 p.m.) [11,33], each exposure lasted approximately 1 h. The total exposure period was 5 uninterrupted weeks (35 days).

The anti-inflammatory efficacy of Ac2-26 the AnxA1 mimetic peptide (Ac-AMVSEFLKQAWFIENEEQEYVQTVK, Thermo Fisher Scientific, Grand Island, NY, USA) in protecting against inflammatory processes caused by exposure to tobacco smoke was evaluated in one of the smoke-exposed groups (n = 10) by the intraperitoneal (ip) administration of Ac2-26 at the dosage of 1 mg/kg in 100 μL of phosphate buffered (PBS) solution [28–33], once a day and before the first exposure to cigarette smoke. The treatment protocol started along with the protocol of exposure to cigarette smoke and had the same duration of 5 weeks.

The control group (n = 10) was kept in the same conditions but

exposed only to compressed air, it was neither exposed to cigarette smoke nor treated [33]. The day after the end of the exposure protocol, the animals were euthanized by excessive dose of anesthetic (thiopental).

2.3. Physiological analyses of plethysmography, blood pressure and weight

On the first and last weeks of the exposure protocol, the rats were evaluated for plethysmography by measuring lung ventilation, breathability, frequency and the inspired volume in a specific apparatus adapted to the use (PowerLab, AD Instruments-Gas Analyzer, Sydney, Australia). In the same periods, the rats were weighed and had the blood pressure measured by the tail in a non-invasive way (PowerLab, TransLower for rats, PanLab, Sydney, Australia).

2.4. X-ray images

Although the parameters of chest X-ray in cases of initial COPD are limited, this methodology is clinically established in cases of moderate or severe disease contributing for COPD diagnostic [34]. We also proceed to X-ray studies, because our research involves systemic and varied evaluations.

After the exposure period (35 days), the animals were anesthetized i.p. with Ketamine (0.8 mL/kg) (BioChimico, Itatiaia, Brazil) and Xylasin (0.2 mL/kg) (Ceva Santé Animale, Paulínea, Brazil). Then, radiographic images were taken of the upper anterior chest region, indicating the airway by macroscopic images of 18 cm wide by 24 cm height of the lungs scanners in a specific device (X-ray - Astex, Vet maq, Brazil) adapted to be used in Wistar rats. The variables considered were the pulmonary retrosternal height of the right lung and the right and left diaphragmatic lowering levels. The perimeter and height measurements were performed by the image analysis software (Leica).

2.5. Quantitative analyses of bronchoalveolar lavage

BAL was obtained at the end of the experiment. The animals had the trachea cannulated and the right lung clamped. The left lung was washed 3 times with 500 μL of PBS and the collected liquid was centrifuged for 10 min at 1500 rpm. The supernatant was stored at −70 °C for subsequent biochemical and cytokine assays. The pellet was resuspended in 500 μL PBS and 10 μL aliquots were stained in Turk (1:10) for quantification of inflammatory cells in a Neubauer camera (values as number of cells × 10^3/mL).

2.6. Histopathological and immunohistochemical studies

After BAL collection, the trachea and right lung were removed, fixed in 4% formaldehyde and processed for paraffin inclusion. Sections of 5 μm were used for histopathological, morphometric and immunohistochemical analyses in a Leica microscope (DM500). For histopathological studies the tissue sections were stained with Hematoxylin and Eosin (HE). Morphometric studies were performed by means of pulmonary alveolar area measurements using an image analyzer (Software Leica Image Analyses).

In the immunohistochemical studies, the expressions of the AnxA1, Cyclooxygenase-2 (COX-2) and MMP-9 proteins and the identification of the macrophages were evaluated. After antigenic recovery with citrate buffer pH 6.0 and blockade of the endogenous peroxidase activity, the sections were incubated with the following rabbit polyclonal primary antibodies: anti-AnxA1 (1: 1000) (Invitrogen, Cat No: 71-3400), COX-2 (1: 300) (Bioss, Cat No: 0732R), MMP-9 (1: 100) (Abcam, Cat No: Ab38898) and ED-1 (for identification of macrophages) (1: 150) (Millipore Corporation, Cat No: MAB 1435, Zymed Laboratories, Cambridge, UK), for 12 h. They were then incubated with the biotinylated secondary antibody (Histostain Kit, Invitrogen, Cat No: 95-9643B) and immersed in conjugated streptavidin peroxidase complex. Substrate diaminobenzidine (DAB Kit, Invitrogen, Cat No: P00-2020) was used for development and, thereafter, the sections were stained with Hematoxylin. Proteins were quantified by densitometry (arbitrary units 0 to 255) using the Leica Image Analysis Software [27,33,35]. The lung macrophages were quantified in 10 random images per slide under the 40× objective of the Leica microscope (DM500) and the tissue areas were obtained in the image analyzer.

2.7. Quantification of mast cells

The mast cells were stained with 0.1% Toluidine Blue and analyzed according to their intact or degranulated morphological characteristics [35]. The amount of histamine in the mast cells was evaluated in the conditions of immaturity and maturity after the respective stains with Alcian Blue (EasyPath, Alcian Blue pH 2.5 PAS, São Paulo, Brazil) and 2.5% Safranin (INLAB Confiança, São Paulo, Brazil) [35,36]. Quantification of mast cells was performed as described for macrophages in the previous item.

2.8. Western blotting studies

The expression of AnxA1 and FPR2 were verified in the supernatants of macerated lungs from all the studied rat groups. Fragments of the left lungs were macerated in liquid nitrogen, and 500 μL of a solution containing protease inhibitors and phosphatase (Merck, Millipore Corporation, USA) were added following the manufacturer's instructions. The material was incubated for 20 min at 4 °C under constant stirring and then centrifuged at 15,000 rpm for 10 min at 4 °C. The supernatants were collected and frozen at −70 °C. The protein concentration in the supernatant was measured using a Bradford assay (Bio-Rad Hercules, USA).

Equal amount samples (40 μg) and molecular weight markers were separated by electrophoresis in 15% polyacrylamide gel according to the Mini-PROTEAN Tetra Cell (Bio-Rad, Hercules, USA) protocol and transferred to nitrocellulose membranes (Hi-Bond C, GE Healthcare, Chicago, EUA).

AnxA1 was detected using the rabbit polyclonal antibody anti-AnxA1 (1:1000) (Invitrogen, Cat No: 71-3400), while fpr2 was detected using the rabbit polyclonal antibody anti-FPR2 (1:2000) (Invitrogen, Cat No: 72-0293). The signal was amplified using the secondary antibody anti-rabbit IgG (1:2000) horseradish peroxidase (HRP)-conjugated (Bio-Rad, Hercules, USA) and the reaction product was visualized in photographic Hyperfilm (GE Healthcare, Chicago, EUA) after the ECL chemiluminescent kit (ECL; GE Healthcare, Chicago, EUA) application. The tubulin was detected as reaction control with the mouse monoclonal antibody IgG, 1:1000 (Thermo Fisher, San Francisco, CA, USA). AnxA1 and fpr2 densitometry was performed using the Leica Image Analysis software.

2.9. Biochemical blood assays

Studies have shown that levels of aspartate aminotransferase (AST), gamma glutamyl transferase (gamma-GT), glucose and cholesterol have been associated with cases of patients with exacerbated COPD [37–40]. Due to this we also investigated these biochemical parameters in the blood.

Right after euthanasia, blood was collected by cardiac puncture in heparinized syringes and separated in aliquots for analysis of hemoglobin with commercial Kit (LAB test, Cat No: 43, Minas Gerais, Brazil). Other aliquots were centrifuged for 15 min at 3000 rpm and the plasma frozen at −70 °C for further biochemical evaluations and cytokine measurements. Total cholesterol (Cat No: 76-2/100), glucose (Cat No: 133-1/500), gamma-GT (Cat No: 105-2/30) and AST (Cat No: 52-200) were measured by means of commercial kits (LAB Test, Minas Gerais, Brazil) in a spectrophotometer (absorbance 540 nm).

2.10. Levels of cytokines

IL-1β, IL-6, IL-10, TNF-α and MCP-1 mediators were quantified in blood plasma, BAL and macerated lung supernatants using the rat cytokine MILLIPLEX MAP Kit (RECYTMAG-65 K; Millipore Corporation, USA) and analyzed on LUMINEX xMAP MAGPIX equipment (Millipore Corporation, USA). The concentration of the analytes was determined by MAGPIX xPONENT software (Millipore Corporation, USA). Results were expressed as mean ± standard error of the mean (S.E.M.) of cytokines concentrations (pg/mL).

2.11. Statistical analysis

Data were analyzed using GraphPad software version 6.00. After analysis of normal distribution the one-way analysis of variance followed by Bonferroni's post hoc test for multiple comparisons was performed to evaluate the mean ± SEM to compare changes in inflammatory cells numbers, morphometry of alveolar spaces, densitometric analysis and MAGPIX values. P values < 0.05 were considered statistically significant.

3. Results

3.1. X-ray images and physiological analyses of plethysmography, blood pressure and weight

Macroscopic lung reductions and elevation of the diaphragm in the inspiratory movement were observed in radiographic images of the untreated smoke-exposed rats (Fig. 1B) compared to the treated (Fig. 1C) and control groups (Fig. 1A). Reduced pulmonary perimeter of the right lung was observed in the treated animals (38.59 ± 1.745 mm) compared to the ones exposed to smoke (41.34 ± 0.6249 mm) (Fig. 1D) and increased level of right diaphragmatic retraction (LRDR) was present in the animals exposed to smoke (5.180 ± 0.9886 mm) compared to the control group (3.178 ± 0.0895 mm) and the treated animals (3.725 ± 0.5737 mm) (Fig. 1E). The final weight analyses showed significant reduction in the CS animals (288.3 ± 5.831 g, $P < 0.01$) in relation to the control group (329.4 ± 2.581 g and Fig. 1F).

The plethysmography analysis showed significant reduction in lung frequency in CS animals (69.54 ± 2.379 Fr/min) compared to control (87.10 ± 3.634 Fr/min, $P < 0.05$) and CS + Ac2-26 animals (95.32 ± 4.178 Fr/min; $P < 0.001$, Fig. 1G). There was no significant difference in inspiratory volume among groups (Fig. 1H). The final ventilation capacity was also reduced in the group exposed to smoke without treatment (762.3 ± 11.53 mL/min) in relation to the control (943.2 ± 34.19 mL/min, $P < 0.05$) and treated (1076 ± 71.90 mL/min, $P < 0.01$, Fig. 1I) rats.

As expected, the pressures at the end of the exposure protocol were significantly increased ($P < 0.001$) in CS animals (80.15 ± 1.722 mm Hg) compared to the control (69.00 ± 1.317 mm Hg) and treated ones (67.22 ± 1.461 mm Hg and Fig. 1J).

3.2. Quantitative studies of bronchoalveolar lavage and histopathological analyses of the trachea and lung

The results of pulmonary histopathological (Fig. 2A–C) and morphometric (Fig. 2G) analyses evidenced tissue preservation in the peptide-treated group. Enlargements of lung intra-alveolar spaces were observed in the group exposed to smoke without treatment (24,771 ± 2929 μm^2) in relation to C (9,668 ± 481.7 μm^2; $P < 0.01$) and CS + Ac2-26 groups (13,235 ± 1427 μm^2; $P < 0.05$). CS animals also showed increased bronchus-associated lymphoid tissue (BALT) (Fig. 2B) and greater influx of inflammatory cells compared to the other groups (Fig. 2A and C).

Histopathological studies of trachea (Fig. 2D–F) indicated changes in the lining epithelium caused by exposure to cigarette smoke with loss of the protection cilia (Fig. 2E) in CS group in relation to control (Fig. 2D) and CS + Ac2-26 (Fig. 6F), in which the preservation of epithelial tissue was observed.

In BAL analysis a significant increase in macrophages ($22 \times 10^5 \pm 2.121 \times 10^5$ macrophages/mL) ($P < 0.001$) (Fig. 2H) and lymphocytes ($47.75 \times 10^5 \pm 11.86 \times 10^5$ lymphocytes/mL) ($P < 0.01$ and Fig. 2I) was observed in the CS group compared to the control (macrophage: $3.250 \times 10^5 \pm 1.652 \times 10^5$ macrophages/mL; lymphocyte: $2.250 \times 10^5 \pm 1.109 \times 10^5$ lymphocytes/mL). However, treated animals showed reduction of these cells (macrophages: $10 \times 10^5 \pm 2.483 \times 10^5$ macrophages/mL, $P < 0.01$, lymphocytes: $20.20 \times 10^5 \pm 3.338 \times 10^5$ lymphocytes/mL, $P < 0.05$).

3.3. Quantitative analysis of inflammatory cells in the lung and trachea

3.3.1. Macrophages

Quantification of macrophages was performed by immunohistochemistry, using the ED-1 antibody and the specificity of this analysis was confirmed by the reaction control. In the lungs, the cells were identified in the intra-alveolar spaces and tissue septa. Numerous macrophages (11.0 ± 4.74 macrophages/mm^2) were observed in the CS group (135.0 ± 54.56 macrophages/mm^2) ($P < 0.001$) compared to the control group (Fig. 3A) but fewer cells were presented in the CS + Ac2-26 (43.75 ± 20.75 macrophages/mm^2) ($P < 0.001$). Similar results were observed in tracheal connective tissue, with a higher amount of macrophages in the untreated animals (67.19 ± 20.76 macrophages/mm^2, $P < 0.00$) in relation to control (24.40 ± 7.917 macrophages/mm^2) and treated groups (26.50 ± 8.023 macrophages/mm^2, $P < 0.001$) (Fig. 3F).

3.3.2. Mast cells

The exposure to smoke promoted significant increase in total (17.00 ± 7.321 mast cell/mm^2, $P < 0.05$) and intact (23.75 ± 1.531 mast cell/mm^2; $P < 0.001$; Fig. 3B) mast cells in the untreated group compared to control (total: 17.00 ± 7.321 mast cell/mm^2; intact: 5.833 ± 4.233 mast cell/mm^2), but there was a significant reduction of total (12.75 ± 5.108 mast cell/mm^2; $P < 0,01$), intact (5.313 ± 1.875 mast cell/mm^2; $P < 0,001$) and degranulated (6.000 ± 3.687 mast cell/mm^2; $P < 0,01$; Fig. 3C) mast cells in the CS + Ac2-26 group compared to CS (degranulated: 26.38 ± 10.18 mast cell/mm^2). Similar data were found in the observations performed on trachea with significant increase in total (17.16 ± 8.077 mast cell/mm^2; $P < 0.05$), intact (9.792 ± 3.393 mast cell/mm^2; $P < 0,05$; Fig. 3G) and degranulated (9.464 ± 3.740 mast cell/mm^2, $P < 0.05$; Fig. 3H) mast cells in CS group compared to the control group (total: 7.800 ± 3.304 mast cell/mm^2, intact: 5.500 ± 2.092 mast cell/mm^2, degranulated: 5.208 ± 2.426 mast cell/mm^2) and treated animals (total: 3.344 ± 2.150 mast cell/mm^2, $P < 0,001$, intact: 1.875 ± 0.6847 mast cell/mm^2, $P < 0.001$, degranulated: 2.708 ± 1.840 mast cell/mm^2, $P < 0.01$).

Mast cells were also quantified following Safranin and Alcian Blue stainings. Increased Alcian Blue ($P < 0.05$; Fig. 3D) and especially Safranin ($P < 0.01$; Fig. 3E) positive mast cells were found in the lungs of the CS group (34.11 ± 18.72 mast cell/mm^2) compared to control (6.750 ± 3.010 mast cell/mm^2) and treated animals (15.00 ± 8.385 mast cell/mm^2). Numerous mast cells, especially Safranin positive ($P < 0.01$), occurred in the trachea of untreated rats (16.46 ± 9.982 mast cell/mm^2) compared to the control ones (2.500 ± 1.976 mast cell/mm^2) with reduction after the peptide administration (2.083 ± 2.041 mast cell/mm^2) (Fig. 3I and J).

3.4. Lung and trachea immunohistochemical studies

Untreated smoke-exposed animals showed increased expression of

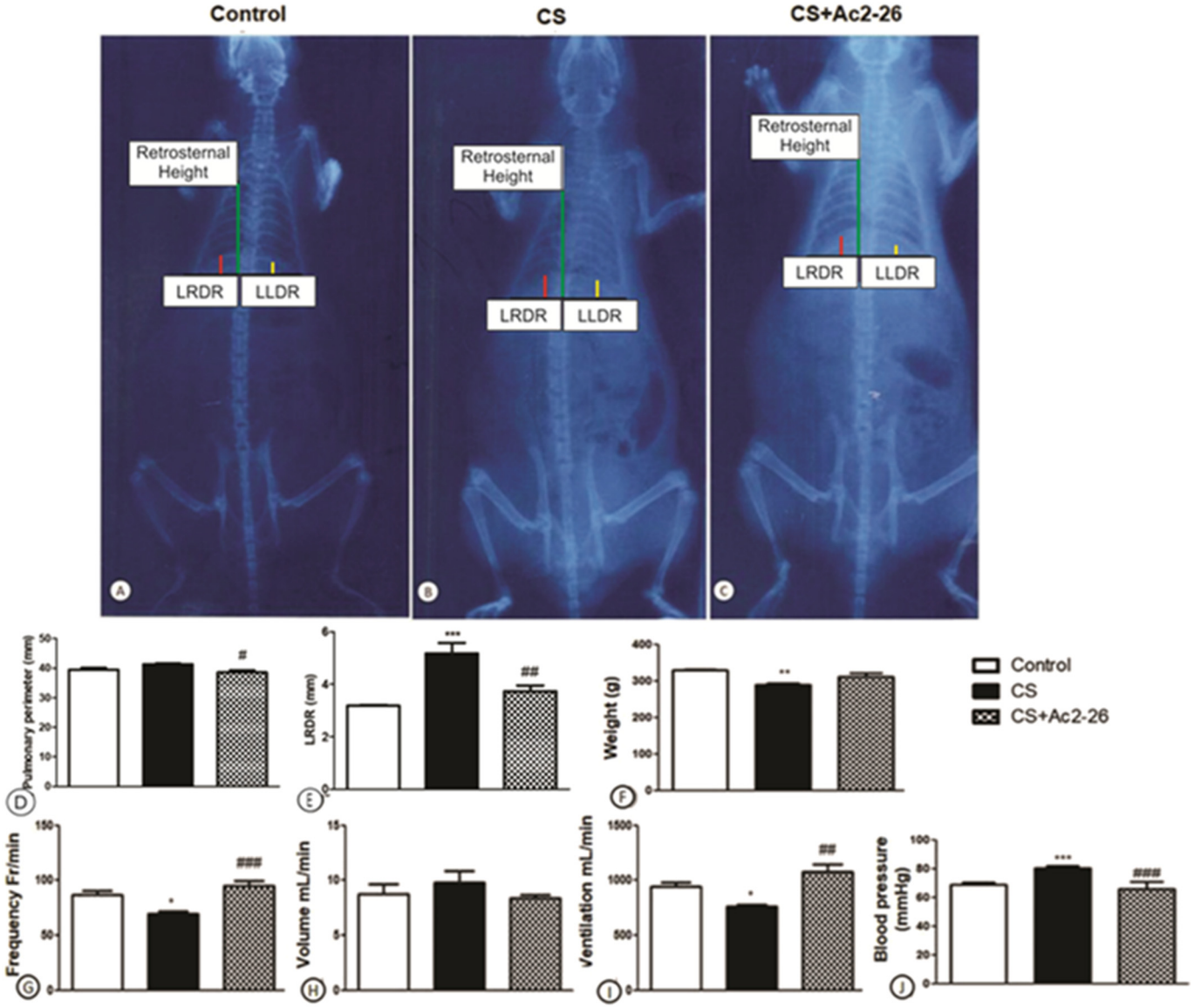

Fig. 1. Radiographic analyses of lungs - Chest regions were evaluated by comparing the lungs among the control (A), untreated and exposed to smoke (CS) (B) and exposed to smoke and treated (CS + Ac2-26) (C) groups. Measure of 18 cm × 24 cm. Variables considered in the X-ray analysis: pulmonary retrosternal height from the beginning to the end of the right lung (Green line - A–C), level of right diaphragmatic retraction (LRDR – Red line - A–C) and level of left diaphragmatic retraction (LLDR – Yellow line - A–C) (mm). Pulmonary perimeter of the right lung (mm) (D) and LRDR (mm) (E) Physiological analyses of plethysmography, blood pressure and weight. Weight of the animals (F). Assessment of lung frequency per minute (G). Capacity of inspired air volume by mL per minute (H), Lung ventilation capacity per minute (I). Blood pressure analyses (mm Hg) (J); Results presented as mean ± S.E.M. (N = 10), * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$ vs control; # $P < 0.05$; ## $P < 0.01$ and ### $P < 0.001$ vs untreated smoke-exposed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

AnxA1 in the lung (189.4 ± 11.54 arbitrary units - a.u., $P < 0.01$, Fig. 4B and M) and trachea (176.1 ± 15.46 a.u., $P < 0.05$, Fig. 4P) compared to control (lung: 169.5 ± 7.238 a.u.; Fig. 4A, trachea: 152.3 ± 14.83 a.u., Fig. 4P). In contrast, the CS + Ac2-26 group indicated reduction in AnxA1 expression in the lungs (167 ± 5.984 a.u., $P < 0,01$, Fig. 4C and M) and trachea (153.8 ± 11.92 a.u.; $P < 0.05$; Fig. 4P).

COX-2 expression was also increased in pulmonary (189.2 ± 7.520 a.u., $P < 0.001$, Fig. 4F and N) and tracheal (182.4 ± 13.94 a.u., $P < 0.05$, Fig. 4Q) tissues in CS animals compared to the control ones (lung: 152.3 ± 8.678 a.u.; Fig. 4E; trachea 156.6 ± 1.790 a.u., Fig. 4Q). Treatment with the peptide reduced the expression of the enzyme in the lungs (164.8 ± 11.99 a.u.; $P < 0.001$; Fig. 4G and N).

Immunoreactivity analyses of MMP-9 indicated significant increase ($P < 0.001$) in lung metalloproteinase expression in untreated (175.6 ± 10.16 a.u.; Fig. 4J and O) and treated animals (176.2 ± 2.831 a.u., Fig. 4K and O) in relation to control group (142.6 ± 7.506 a.u.; Fig. 4I and O). A reduction in MMP-9 expression ($P < 0.001$) in the trachea was observed after treatment with the peptide (159.9 ± 12.94 a.u.; Fig. 4R) compared to the untreated animals (171.7 ± 4.777 a.u.).

Expressions of the AnxA1, COX-2 and MMP-9 proteins were analyzed in the lung and trachea of all groups. The specificity of the immunolabeling was confirmed by the respective reaction controls (Fig. 4D, H and L).

3.5. Western blotting analysis

Densitometric analyses indicated that cigarette smoke exposure

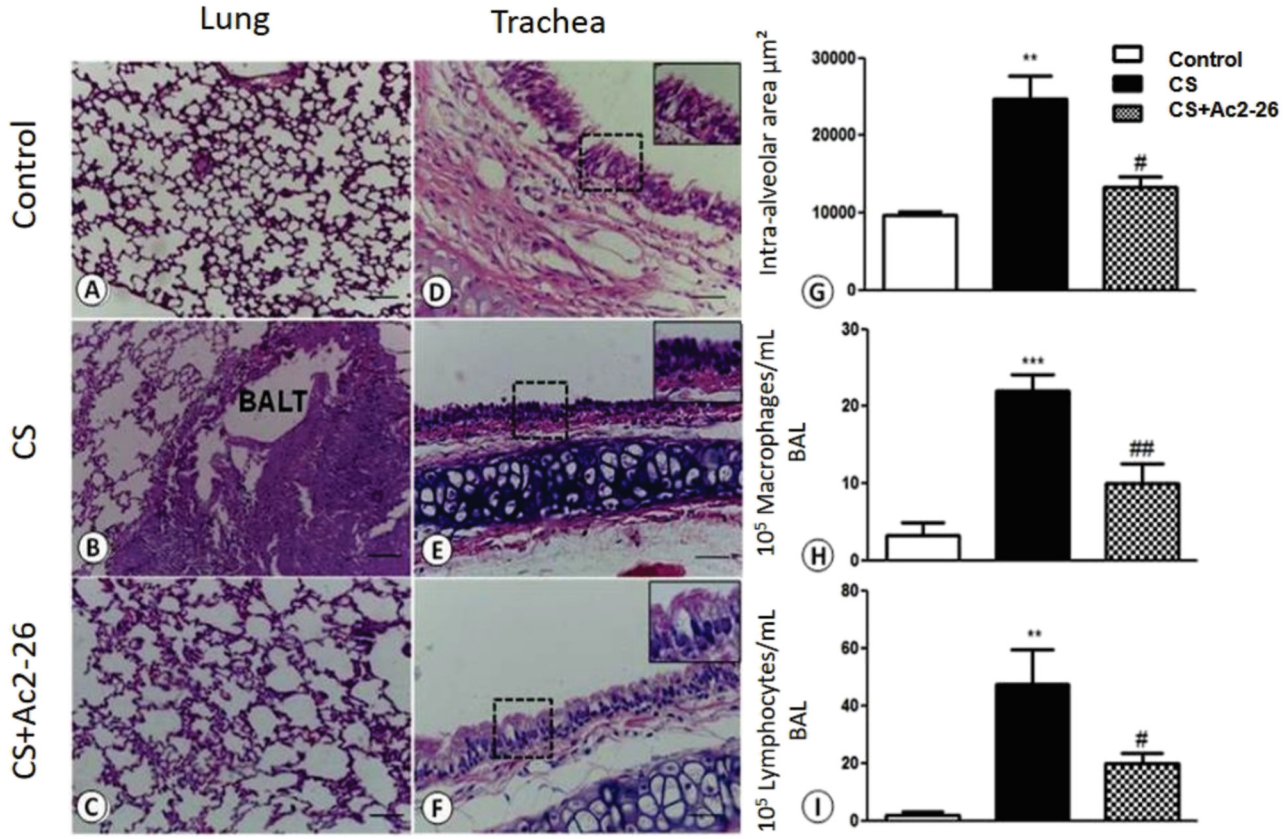

Fig. 2. Histopathological analysis of lung - Control group (A), untreated-exposed to smoke group (CS) with increased intra-alveolar spaces and Bronchus associated lymphoid tissue (BALT) (B) and smoke-exposed animals treated with the peptide (CS + Ac2-26) (C). Color: Hematoxylin-Eosin. Bars: 10 µm. Morphometry of intra-alveolar spaces (µm^2) (G). Results presented as mean ± S.E.M. (n = 10/group). Histopathological analysis of trachea- Control (D), untreated and smoke-exposed group with tissue alteration and loss of cilia (E) and treated with peptide group with preservation of epithelial tissue (F). Color: Hematoxylin-Eosin. Bars: 10 µm. Quantitative analysis of bronchoalveolar lavage (BAL) - Quantification of macrophages (H) and lymphocytes (I) in a Neubauer camera. Results presented as mean ± S.E.M. (n = 10), ** $P < 0.01$ and *** $P < 0.001$ vs control; # $P < 0.05$ and ## $P < 0.01$ vs untreated smoke-exposed.

upregulated AnxA1 expression but treatment with Ac2-26 significantly reduced the levels of intact form of AnxA1 ($P < 0.001$). Interestingly, we found FPR2 overexpression ($P < 0.001$) in the group treated with the mimetic peptide of AnxA1 (Fig. 5).

3.6. Biochemical blood assays

Hemoglobin concentration was elevated ($P < 0.01$) in the CS animals (16.92 ± 1.224 g/dL) in relation to control rats (10.23 ± 1.229 g/dL) (Fig. 6A). Regarding the glucose (Fig. 6B) and Gamma Glutamyl Transferase (Gamma-GT) measurements (Fig. 6C), higher dosages ($P < 0.05$) were observed in the CS (glucose: 428.7 ± 35.85 mg/dL and gamma-GT: 15.97 ± 5.559 U/L) compared to control (glucose: 169.1 ± 4.695 mg/dL and gamma-GT: 7.644 ± 2.049 U/L) and significant reduction ($P < 0$, 01) after the treatment (4.860 ± 3.519 U/L). Similarly, decreased cholesterol (Fig. 6D) and Aspartate aminotransferase (AST) ($P < 0.05$) (Fig. 6E) levels were observed in CS + Ac2-26 animals (Cholesterol: 65.02 ± 18.33 mg/dL and AST: 111.2 ± 8.565 U/mL) compared to the untreated group (Cholesterol: 91.91 ± 26.70 mg/dL and AST: 127.8 ± 9.230 U/mL).

3.7. Cytokines concentrations

In the macerated lung supernatant of CS animals, higher levels of the pro-inflammatory mediators IL-1β ($P < 0.01$), IL-6, TNF-α ($P < 0.001$) and MCP-1 ($P < 0.001$) were observed in relation to the control group animals (Fig. 7A, B, D and E). Likewise, there were increases in IL-1β, IL-6 ($P < 0.001$) and MCP-1 ($P < 0.01$) in the blood plasma (Fig. 7F, G and I) and also of IL-1β ($P < 0.001$) and MCP-1 ($P < 0.01$) in BAL (Fig. 7K and N).

The peptide administration promoted significant reduction of the pro-inflammatory mediators levels in the supernatant (IL-1β and MCP-1 $P < 0.01$, IL-6 and TNF-α $P < 0.001$) (Fig. 7A, B, D and E), blood plasma (IL-1β and IL-6 $P < 0.001$; TNF-α $P < 0.01$) (Fig. 7F, G and J) and BAL (IL-1β $P < 0.001$ and TNF-α $P < 0.05$) (Fig. 7K and O). Differently, in the untreated smoke-exposed animals, reduced levels of the anti-inflammatory cytokine IL-10 were observed in BAL ($P < 0.01$) in relation to the control group (Fig. 7M). Besides increased IL-10 levels were found in macerated lung supernatant and BAL of treated animals ($P < 0.001$) (Fig. 7C and M).

4. Discussion

Smoking is strongly associated with the development of COPD, a disease induced by inflammatory process and characterized by progressive limitation of airflow [2,13]. For these reasons we investigated the effects of the anti-inflammatory protein AnxA1 mimetic peptide administration in a model of initial COPD by exposure to cigarette smoke in rats.

Initially the physiological data were analyzed and, as expected, there were weight loss, increased blood pressure and reduction of the frequency and ventilation capacity in rats exposed to smoke without treatment. In addition, our radiographic imaging data showed reductions in the pulmonary perimeter of the untreated smoke-exposed animals compared to the control and peptide treated smoke-exposed

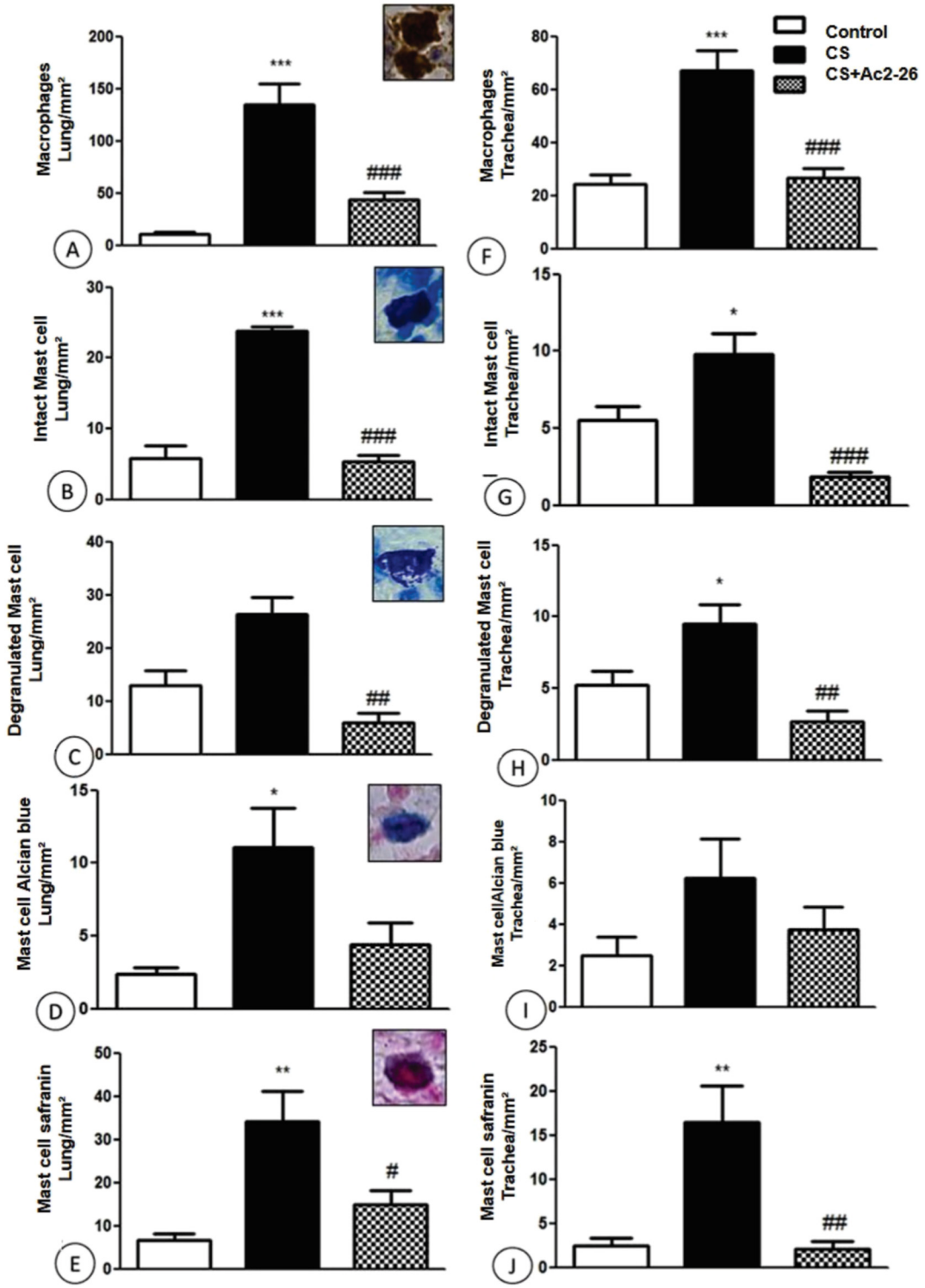

Fig. 3. Macrophages and mast cells in lung and trachea - Few macrophages (A and F) and mast cells (B–E, G–J) in the control group. Increase in these cells in the group exposed to smoke without treatment and reduction after treatment with the peptide. (n = 10/group). Counter-staining: Hematoxylin. Color: Toluidine blue (B, C, G and H), Alcian blue (D and I) and Safranin (E and J). 10 μm bars. Results presented as mean ± S.E.M. * P < 0.05; ** P < 0.01; ** P < 0.01 *** P < 0.001 vs control; # P < 0.05; ## P < 0.01; ### P < 0.001 vs untreated smoke-exposed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

groups. Higher blood pressure and weight loss are commonly observed in patients with COPD [1,4]. The impact of cigarette smoke on weight gain was also observed in another research using a rat-COPD model of 5 weeks exposure to cigarette smoke [10]. Concerning the respiratory alterations, studies indicate that patients with thicker lung walls related to emphysema or COPD present severe clinical conditions and airflow limitation with compromised lung function [41]. Our results point that airflow limitation occurs due to the reduction of the airway diameter at low pulmonary level and are in line with other investigations [42,43]. Moreover, in the animals treated with the peptide, the weight, blood

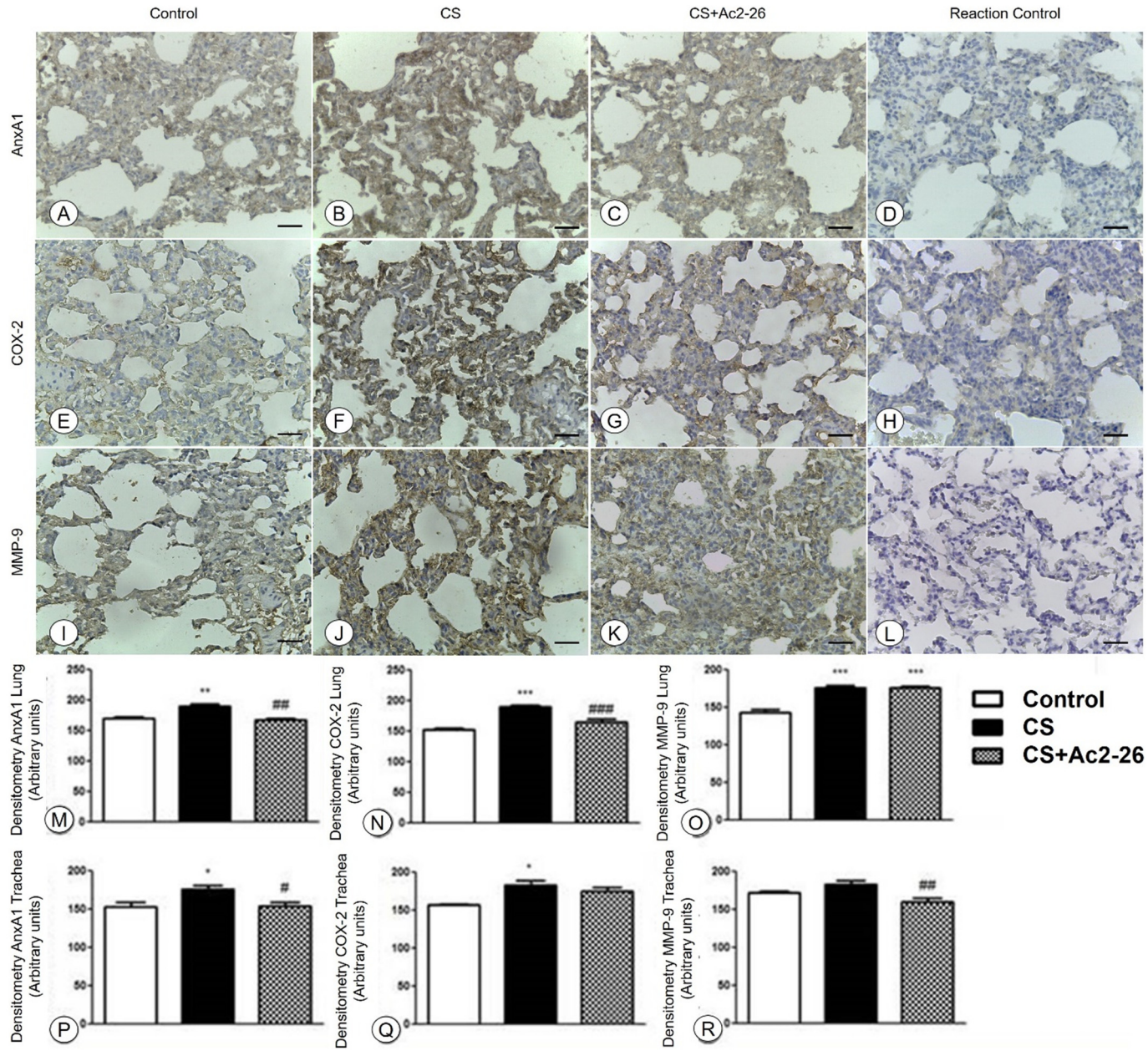

Fig. 4. Expression of the AnxA1, COX-2 and MMP-9 proteins in lung and trachea: Reduced expression of the proteins in the control group (A, E and I), increased immunolabeling in the untreated smoke-exposed group (CS) (B, F and J) and reduction in the expression of AnxA1 and COX-2 after treatment with the peptide (CS + Ac2-26) (C and G). Absence of immunolabeling on reaction control (D, H and L). Counter-staining: Hematoxylin. 10 μm bars. Results presented as mean ± S.E.M. (n = 10/group) (M, N, O, P, Q and R). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs control; # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ vs untreated smoke-exposed.

pressure, ventilation and lung dimensions were similar to those found in control animals, showing the beneficial effects of the treatment against the systemic damages of smoking exposure. These data are in agreement with studies that demonstrate the protective role of Ac2-26 in pulmonary diseases such as lung inflammation, fibrosis and silicosis [28–31].

A recent research showed upregulated expression of AnxA1 in cases of severe COPD related to lung fibroblast activation, indicating that AnxA1 may be involved in airway fibrosis of COPD [44] These authors suggest that AnxA1 is increased in COPD patients in an attempt to attenuate the exacerbated inflammatory response but the resolution of the inflammation is dysfunctional and the elevated AnxA1 expression is insufficient to resolve inflammation. These findings reinforce the importance of our study on the administration of Ac2-26 in an animal model of COPD. Our results indicated that in the initial phase of COPD, the Ac2-26 peptide of AnxA1 assists in reducing the inflammatory process.

To complement our physiological studies we proceeded with the blood biochemical assays. Although studies showed that the hemoglobin level in COPD patients is low, here we found higher concentration of hemoglobin only in the untreated smoke-exposed group, which corroborates another study that indicated increased hemoglobin level in mice exposed to short-term cigarette smoke [45]. As the model we used here can be considered as initial COPD, the increased hemoglobin level may be a compensatory mechanism in view of the higher concentration of carbon monoxide. The hemoglobin increase can be associated with elevated serum erythropoietin (EPO), a glycoprotein that stimulates red cell production [46], in the initial phase of COPD

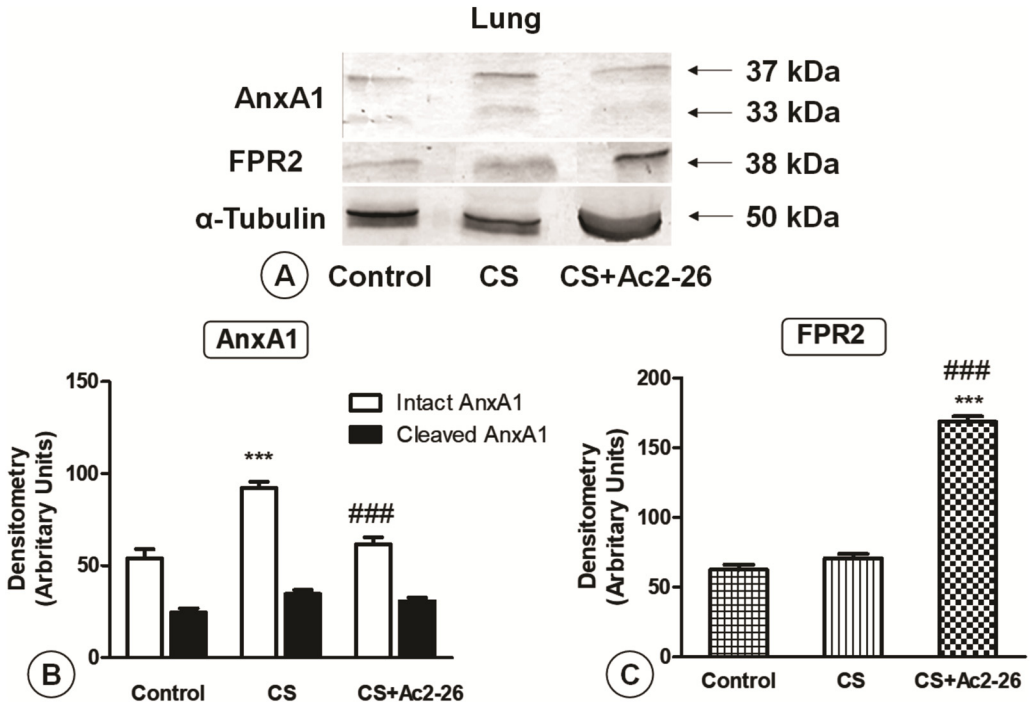

Fig. 5. AnxA1 and FPR2 expressions in lung macerate supernatants - Representative Western blotting illustrating the intact (37 kDa) and N-terminal – cleaved (33 kDa) forms of AnxA1 and FPR2 (n = 3 blots/group). Equal loading was confirmed with anti–a-tubulin. (A) Blots are representative of at least three separate experiments with similar results. Densitometric analyses of AnxA1 (B) and FPR2 (C). Results presented as mean ± S.E.M. (n = 10/group). *** P < 0.001 vs control; ### P < 0.001 vs untreated smoke-exposed.

although EPO may be reduced in cases of exacerbation of COPD [47].

In the other biochemical analyses, we observed elevated glucose and cholesterol levels in the untreated smoke-exposed group compared to the control group and significant reduction after treatment. COPD patients have higher prevalence of diabetes mellitus. Besides, the elevated blood glucose levels associated with increased oxidative stress and inflammation can affect the pulmonary function [40]. The association between the amount and duration of smoking on biochemical, clinical parameters and the ovarian morphology in women with polycystic ovary syndrome also indicate increase in total and low-density lipoprotein (LDL) cholesterol in smokers compared to nonsmokers, pointing that increase of smoking aggravates the lipid profile [48].

Augmented levels of AST and gamma-GT were also observed in the untreated smoke-exposed group in our studies. Other research showed significant increase in biochemical parameters related to liver function, as serum levels of alanine aminotransferase (ALT), AST and total cholesterol were significantly higher in mice that were short-term exposed to cigarette smoke [39]. Moreover smokers with elevated blood pressure and serum gamma-GT levels are at higher risk of developing chronic kidney disease [49]. Other researches pointed increased gamma-GT values in COPD patients indicating that serum GGT activity might also represent an inflammation/oxidative stress marker [37], and also that there is a strong correlation between high gamma-GT serum levels and cardiovascular events in COPD patients [38]. Again the reversal of AST and gamma-GT levels with Ac2-26 administration reinforces the protective potential of treatment with Ac2-26.

Following, our histopathological analyses showed enlarged intra-alveolar spaces, lung parenchyma degeneration, influx of inflammatory

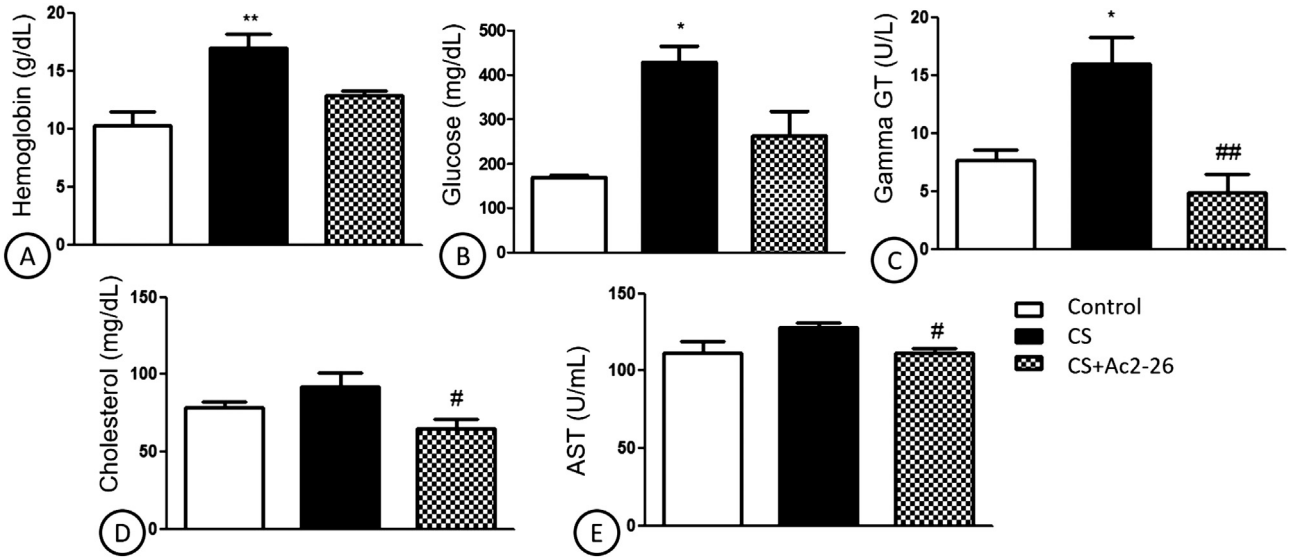

Fig. 6. Biochemical analyses of blood - Hemoglobin (A) was measured in blood per g/dL. The dosages of glucose (B), gamma GT (C), cholesterol (D) and AST (E) were performed in blood plasma, respectively per mg/dL, U/L and U/mL. Results presented as mean ± S.E.M. (n = 10/group). * P < 0.05; ** P < 0.01 vs control # P < 0.05; ## P < 0.001 vs untreated smoke-exposed.

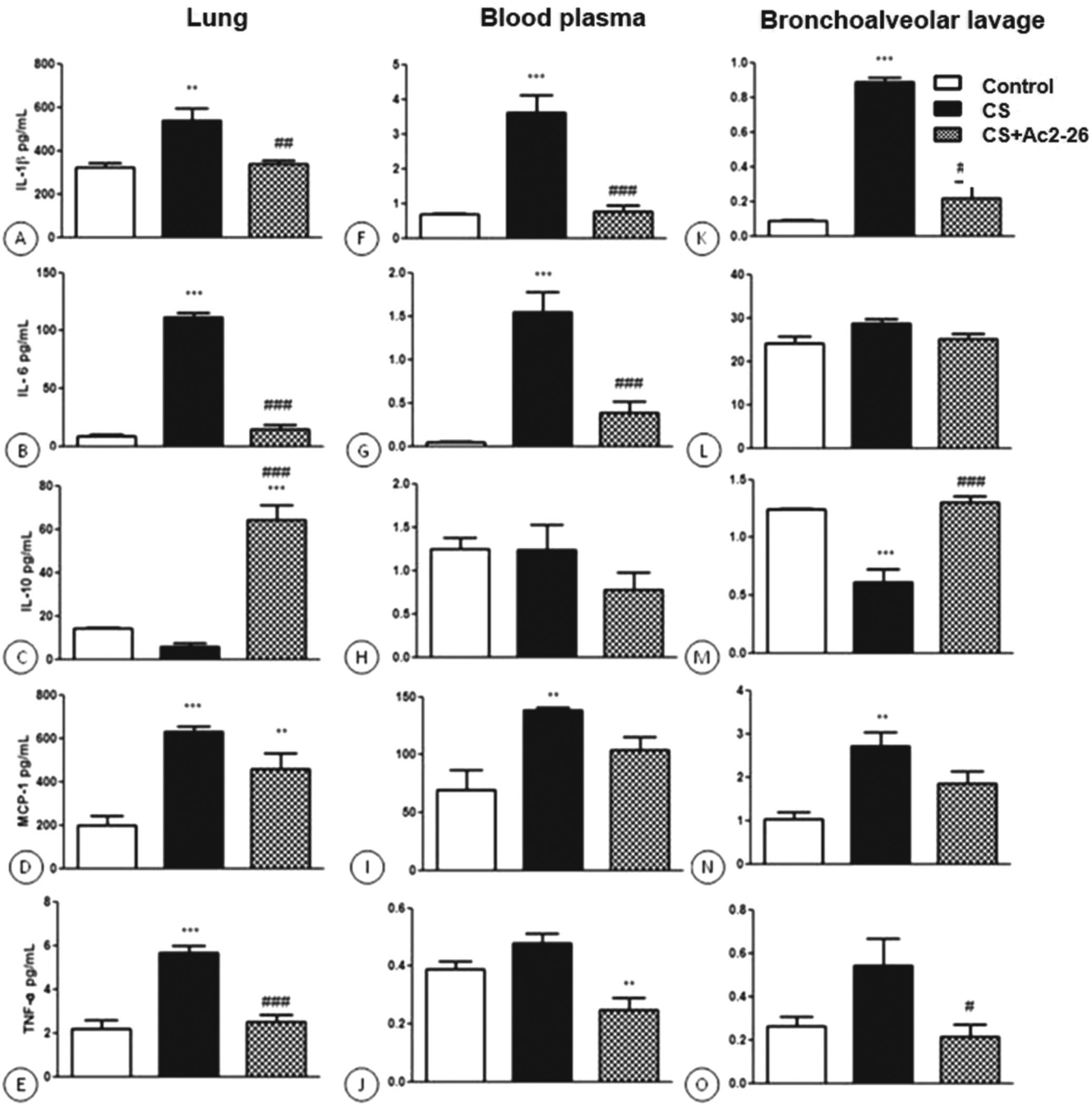

Fig. 7. Cytokine levels in lung macerate supernatant, blood plasma and bronchoalveolar lavage - IL-1β (A, F and K); IL-6 (B, G and L); IL-10 (C, H and M); MCP-1 (D, I and N); TNF-α (E, J and O). Results presented as mean ± S.E.M. (n = 10/group). ** P < 0.01; *** P < 0.001 vs control # P < 0.05; ## P < 0.01 ### P < 0.001 vs untreated smoke-exposed.

cells and an increase in BALT mainly in the untreated smoke-exposed group. The reduction of airflow and consequent difficulty in breathing may be related to the imbalance between the proteases responsible for the destruction of the pulmonary parenchyma and antiproteases that inhibit the action of the proteolytic enzymes [32,50]. However, minor changes occurred in rats treated with the peptide Ac2-26 that showed preservation of the pulmonary parenchyma and reduced inflammatory cells. According to another study, the pre-treatment with Ac2-26 inhibited leukocytes migration in the experimental model of lung inflammation induced by LPS [29].

The anti-inflammatory role of Ac2-26 was also observed by our analyses of inflammatory cells in BAL, which showed higher amount of lymphocytes and monocytes in untreated smoke-exposed animals and reduction after treatment. The increase in leukocytes after exposure to cigarette smoke, especially in the untreated group, is in accordance with investigations that correlate the elevated number of inflammatory cells in bronchial biopsies and expectoration to the severity of the disease with a decline in lung function and health status in the COPD [3]. The reduction of the leukocyte influx promoted by Ac2-26 was also indicated in other experimental models of inflammation [20,24,27] and confirmed the anti-inflammatory potential of the peptide once again. The shedding of L-selectin promoted by AnxA1 and its mimetic peptide leads to the detachment of leukocytes and reduces cell transmigration [20,23,24].

In our tissue quantifications of inflammatory cells, numerous macrophages were identified by immunohistochemistry in the intra-

alveolar spaces and tissue septa as well as in tracheal connective tissue of the smoke-exposed group without treatment, corroborating other studies that showed increase in these cells and the development of airway dysfunction in the smoke-exposed groups [14]. The activation of macrophages and neutrophils in small airways leads to tissue damage by the release of toxic mediators, such as oxidants and proteolytic enzymes [13]. Differently, treatment with Ac2-26 was able to reduce the number of macrophages in both lung and trachea, reinforcing the anti-inflammatory role of the peptide in COPD.

Our studies also revealed numerous mast cells in the lung and trachea and they were found intact and degranulated, as well as Alcian Blue and mainly Safranin positive in the untreated smoke-exposed group. However, a marked reduction in these cells occurred by Ac2-26 administration. Other investigations demonstrate that smoking increases the production of histamine by alveolar mast cells with subsequent degranulation, which contributes to destruction of the alveolar septa, recruitment of neutrophils and release of cytokines and chemokines [51–53].

After evaluating the anti-inflammatory activity of the exogenous AnxA1, we studied the expression of the endogenous protein and we found higher AnxA1 expression in the lung and trachea tissues and also in lung supernatants of the untreated smoke-exposed group, indicating the participation of the endogenous protein in the control of inflammation and tissue preservation. In the treated smoke-exposed group a reduction in the protein expression was observed, pointing the anti-inflammatory action of Ac2-26. The reduction in the expression of the endogenous AnxA1 is possibly associated with negative feedback process [27]. Knowing that the AnxA1 protein binds to the receptor FPR we investigated the FPR2 receptor expression in the macerated lung supernatant. Our results indicated increased FPR2 expression in the untreated smoke-exposed group and overexpression by the Ac2-26 treatment. These findings support the idea that AnxA1 and Ac2-26 act through the FPR receptor. Similar results were found by our research group in a model of endotoxin induced uveitis [27], in which the anti-inflammatory role of Ac2-26 was abrogated by the nonselective FPR antagonist, Boc2. The expression of AnxA1 and its relation to FPR receptors were also investigated in the respiratory tract [31,54,55]. AnxA1 and FPR2 levels in lung tissue and BAL were significantly higher in mice sensitized and challenged with ovalbumin compared with control animals [55]. Increased lung edema, neutrophil infiltration, inflammatory cytokine release, oxidative stress, apoptosis, translocation of nuclear factor (NF)-κB and tissue damage were attenuated by Ac2-26 and FPR activation in acute lung injury induced by Ischemia-Reperfusion in rats, but the protective effect of Ac2-26 administration was reduced by Boc2 [54]. The lack of AnxA1 exacerbated the inflammatory and fibrotic responses and altered lung function in silica-exposed mice [31], but the Ac2-26 treatment significantly reduced the production of cytokines and reduced the inflammatory response. Moreover, in vitro analyses pointed that FPR1 and FPR2 mediate inhibition of lung fibroblast activation by peptide Ac2-26 [31].

As AnxA1 can regulate the COX-2 enzyme in inflammatory processes [27] we, then, proceeded with the expression analysis of this enzyme. We found increased COX-2 immunoreactivity in the lung and trachea in untreated smoke-exposed animals that was attenuated by Ac2-26 administration. Increased expression of COX-2 was associated with the high degree of inflammation in COPD [56]. Moreover, studies with culture of lung tumor cells reported that the anti-inflammatory activity of green tea (*Camellia sinensis*) is mediated by AnxA1 overexpression and reduction in COX-2 and prostaglandin 2 [57].

In addition to the regulatory role on COX-2 [27], AnxA1 was strongly characterized as a positive regulator of MMP-9, via NF-kβ pathway in cases of breast cancer, presenting a protective role against the invasion of cancer cells [58]. In another study, the Ac2-26 peptide was able to reduce the expression of the *MMP-2* and *MMP-9* genes in Hep-2 tumor cells (derived from laryngeal epidermal carcinoma) [59]. In view of this and knowing that other investigations associated several MMPs, including MMP-9, with tissue changes in patients with pulmonary emphysema [15,56] the expression of this metalloproteinase was analyzed in the continuity of the immunohistochemical studies. We identified higher MMP-9 immunolabeling in the lungs and trachea in the untreated smoke-exposed group, indicating the participation of this enzyme in tissue destruction. However, the peptide treatment was able to reduce MMP-9 expression in trachea, showing again its beneficial and protective effects.

Finally and understanding the importance of the inflammatory mediators in COPD [13] we proceeded with the analysis of pro-inflammatory mediators IL1β, IL-6, TNF-α, MCP-1 and the anti-inflammatory cytokine IL-10 in the macerated lung supernatant, blood plasma and BAL. Our results showed elevated levels of IL-1β, IL-6 and TNF-α in untreated smoke-exposed rats and are in line with other studies indicating the high levels of these cytokines in asthmatic and/or COPD patients exposed to smoke [17]. Thus, the reduction of these chemical mediators in the group exposed to smoke and treated with the mimetic peptide highlights the protective role of Ac2-26 once more. Similarly, the data obtained from the MCP-1 analysis pointed increased chemokine concentration in lungs of untreated smoke-exposed rats and reduced levels in treated smoke-exposed animals. This way, they are consistent with the quantification of lung macrophages in this investigation and with other studies that showed that prolonged smoking habit promotes increase in MCP-1 and contributes to inflammatory profiles [13,16].

Confirming the protective effects of Ac2-26 administration, we identified increased IL-10 levels in the treated smoke-exposed group. The low concentration of this cytokine in animals exposed to smoke without treatment indicates that this mediator is affected by exposure to cigarette smoke [60]. The administration of the AnxA1 mimetic peptide in a model of intestinal ischemia-reperfusion was beneficial, with attenuation of leukocyte migration to the lung and induction of IL-10 release into the blood [28,30]. Similar to our results, the protective effect of Ac2-26 was shown in a model of pulmonary endotoxemia by LPS administration [29], in which the pre-treatment with the peptide regulated the endotoxemic inflammation by decreasing the leukocyte extravasation to the connective tissue and alveolar cavity, reducing the release of TNF-α, IL-6 and IL-1β and increasing the IL-10 in BAL and blood plasma.

Our results all together emphasize the involvement of AnxA1 in the respiratory tract under normal conditions and during the inflammatory stimulus and especially highlight the potential therapeutic use of its mimetic peptide Ac2-26 in the diseases caused by smoking, mainly in the protection against COPD.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2018.08.011.

Acknowledgement

This work was supported by CAPES, FAPESP (grants 2015/03359-5 APG and 2016/020123-4 SMO) and CNPq (grants 308144/2014-7 SMO).

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